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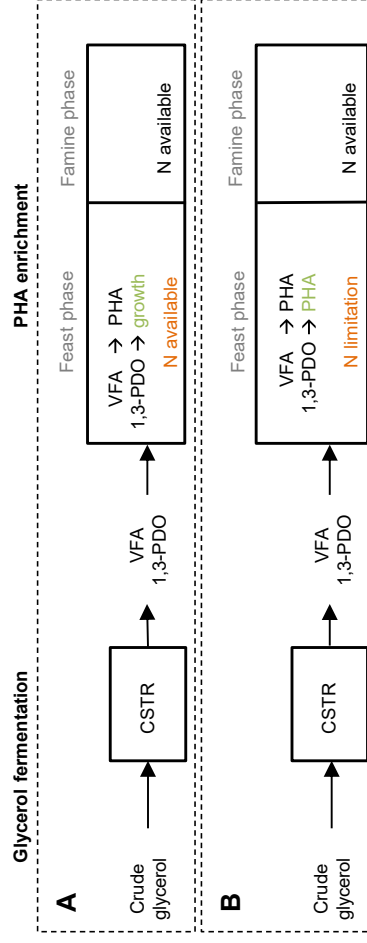
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Title

Polyhydroxyalkanoates (PHA) production from fermented crude glycerol: Study on the conversion of 1,3-propanediol to PHA in mixed microbial consortia

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Abstract

Crude glycerol, a by-product from the biodiesel industry, can be converted by mixed microbial consortia into 1,3-propanediol (1,3-PDO) and volatile fatty acids. In this study, further conversion of these main products into polyhydroxyalkanoates (PHA) was investigated with the focus on 1,3-PDO. Two different approaches for the enrichment of PHA accumulating microbial consortia using an aerobic dynamic feeding strategy were applied. With the first approach, where nitrogen was present during the whole cycle, no net production of PHA from 1,3-PDO was observed in the fermented effluent, not even in a nitrogen-limited PHA accumulation assay. Nevertheless, experiments in synthetic substrates revealed that the conversion of 1,3-PDO to PHA was possible under nitrogen limiting conditions. Thus, a different enrichment strategy was formulated where nitrogen was limited during the feast phase to stimulate the storage response. Nitrogen was still supplied during the famine phase. With the latter strategy, a net production of PHA from 1,3-PDO was observed at a yield of $0.24 \text{ C}_{\text{mol}} \text{ PHA/C}_{\text{mol}} \text{ 1,3-PDO}$. The overall yield from the fermented effluent was $0.42 \text{ C}_{\text{mol}} \text{ PHA/C}_{\text{mol}}$ substrate. Overall, the PHA yield from 1,3-PDO seemed to be limited, similarly to when using glycerol as a substrate, by a decarboxylation step and accumulation of other storage polymers such as glycogen, and possibly, lipid inclusions.

Keywords

Polyhydroxyalkanoates; PHA; crude glycerol; 1,3-propanediol; butyrate; mixed microbial consortia.

1 Introduction

When exposed to unbalanced growth conditions, some bacteria have the ability to store carbon and energy in the form of intracellular compounds such as lipids (e.g. triacylglycerols and wax esters), polyphosphates, carbohydrates (e.g. glycogen) or polyhydroxyalkanoates (PHA) (Dias et al., 2006; Thomson et al., 2010). PHA are polyesters described in more than 300 microorganisms with a diverse monomer composition, although the most commonly reported polymers are polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) (Dias et al., 2006). PHA present similar properties to polyethylene and polypropylene, and therefore they have been proposed as a possible renewable and biodegradable bio-based alternative to these petroleum-derived plastics (Laycock et al., 2014). Nevertheless, the market size is still limited, mostly due to the elevated costs of PHA production. The use of waste substrates and/or mixed

microbial consortia are of increasing interest as strategies to reduce production cost (Laycock et al., 2014; Serafim et al., 2008).

Crude glycerol is among the substrates recently tested for PHA production. This by-product of the biodiesel industry represents 10% of the biodiesel output and contains on average 70% glycerol, among other impurities such as methanol, salts or fats (Zhu et al., 2013). Limited research has been conducted so far in respect to the production of PHA from mixed microbial consortia using crude glycerol as a substrate (Dobroth et al., 2011; Freches and Lemos, 2017; Moita et al., 2014). In synthetic glycerol, Moralejo and colleagues (Moralejo-Gárate et al., 2011) obtained a PHB content of 67% of the cell dry weight and surpassed the results attained with pure strains in terms of specific productivity and yield, reaching these parameters values of 0.34 g PHA/g biomass.h and 0.57 C_{mol} PHA/ C_{mol} S, respectively.

Nevertheless, the production of PHA from glycerol has three main drawbacks: 1) the side production of glycogen (Moita et al., 2014; Moralejo-Garate et al., 2013), 2) the reduced molar mass of the polymer due to glycerol chain termination (Zhu et al., 2013) and 3) the production of HB as only monomer, which has less desirable thermal properties than PHB copolymers (Laycock et al., 2014; Zhu et al., 2013). For these reasons a three step process was chosen in the present work, where a fermentation of glycerol into volatile fatty acids (VFA), recognized as preferred substrates for PHA production, was performed prior to the culture selection and PHA accumulation (Serafim et al., 2008). To our knowledge, this strategy has only been tested once using crude glycerol as a substrate (Shen et al., 2014). Nonetheless, it has never been tested in fermentation effluents containing 1,3-propanediol (1,3-PDO), a major metabolite of glycerol fermentation. As a matter of fact, production of PHA from 1,3-PDO has rarely been studied and never described in mixed consortia. Such conversion has only been reported in recombinant bacteria (e.g. Meng et al., 2012) and in two defined strains producing a mixture of medium and long chain PHA and poly(3-hydroxybutyrate-co-3-hydroxypropionate) (Kimura et al., 2002; Lee et al., 1995), but no conversion yields have been reported.

The most efficient enrichment strategy so far developed for the PHA production in mixed consortia is the aerobic dynamic feeding (ADF), based on the transient limitation of substrate in feast/famine cycles performed in sequential batch reactors (SBR) (Dias et al., 2006; Serafim et al., 2008). The availability of nitrogen during the famine phase is crucial for PHA-accumulating microorganisms to proliferate during the famine phase and overgrow non-accumulating bacteria (Albuquerque et al., 2007). Conversely, PHA yields are generally higher in the absence of nitrogen during the PHA accumulation phase (Johnson et al., 2010a; Marang et al., 2014; Serafim et al., 2004). This discrepancy, together with the incapacity of certain bacteria to accumulate PHA in

the presence of nitrogen (Lee, 1996), set the ground for an alternative enrichment strategy. Nitrogen limitation was applied during the feast phase to promote PHA accumulation, while nitrogen was supplied for growth during the famine phase. The same strategy was very recently reported to increase the PHA yield and productivity using cheese whey as a substrate (Oliveira et al., 2016).

This study focuses on the production of PHA from the two main substrates of the glycerol fermentation: VFA and 1,3-PDO, and evaluates the results obtained from a conventional ADF enrichment strategy and an alternative enrichment strategy based on the nitrogen limitation during the feast phase.

2 Materials and methods

2.1.1 Crude glycerol fermentation

Crude glycerol fermentation run in a 2.5 L Minifors reactor (INFORS HT) with a working volume of 1.7 L operated under continuous mode (CSTR). The reactor ran at a hydraulic retention time (HRT) of 12 h at 37°C and an agitation of 150 rpm under non-sterile anaerobic conditions. The pH was maintained at 5.5 with 4 M KOH. The feed medium consisted of BA (Basic Anaerobic) growth medium supplemented with crude glycerol to a final glycerol concentration of 10 g/L (14.74 g crude glycerol/L). Both the medium and the crude glycerol characteristics are reported elsewhere (Varrone et al., 2015). The reactor was inoculated (10% v/v) with anaerobic sludge previously submitted to an enrichment process (Varrone et al., 2015), and later cultivated under the abovementioned CSTR conditions for 116 HRT (Varrone et al., in press). Nitrogen in the feed was progressively lowered from a C:N:P ratio of 100:5.7:0.5 to a ratio of 100:1.3:0.5 to obtain an effluent with less than 10 mg/L N-NH₃ (2.56 ± 1.74). Distribution of products in the steady state was as follows (g/L): 1,3-PDO: 4.15 ± 0.52 , butyrate: 1.66 ± 0.2 , propionate: 0.78 ± 0.26 , acetate: 0.19 ± 0.07 , glycerol: 0.18 ± 0.21 , TSS: 0.89 ± 0.21 . Measured metabolites accounted for an average of 11.6 ± 0.4 g COD/L, while the total COD was around 14 g COD/L. The effluent was kept at 4°C and at -20°C for short and long term storage, respectively. Before PHA production, it was centrifuged (15 min at 4000 g) and filtered (1µm).

2.2 Sequential batch reactors (Enrichment of PHA accumulating microorganisms)

Enrichment of PHA accumulating microorganisms was performed in the same reactors as the fermentation, but operated in a Sequential Batch Reactor (SBR) mode under a feast-famine regime and non-

sterile conditions. Two types of enrichments were evaluated, one with nitrogen availability during the whole cycle (SBR_N) (Fig. 1A), and another one with nitrogen limitation during the feast phase (SBR_noN) (Fig. 1B).

2.2.1 *Enrichment under nitrogen availability (SBR_N)*

The reactor was initially inoculated with 30% inoculum consisting in equal parts of anaerobic sludge from Lundtofte wastewater plant (Lyngby, Denmark) and aerobic secondary sludge from Daka EcoMotion Biodiesel wastewater treatment plant (Løsning, Denmark). Every 12 h, half of the volume of the reactor (850 mL) was replaced with fresh medium using timer-controlled external pumps. No settling phase was allowed, attaining a HRT and solids retention time (SRT) of 1 day. Aeration was supplied at a rate of 1 L/min with an agitation of 500 rpm. pH was maintained at 8 with 1 M NaOH and 0.5 M HCl. Temperature was set at 30°C. The reactor and the tubing material were emptied and cleaned once a week to avoid excessive biofilm formation.

The medium was prepared with clarified fermentation effluent diluted with distilled water (320mL of effluent per L of medium on average) to obtain a concentration of 90 C_{mM} (mM of carbon) (45 C_{mM} at the beginning of the cycle), which resulted in an organic loading rate (OLR) of around 4.4 g COD_{total}/L/day. Nitrogen and phosphorous were initially adjusted to a C:N:P molar ratio of 100:8:1 with NH₄Cl and K₂HPO₄ and later on to a molar ratio of 100:12:1.5. Allylthiourea (5 mg/L) (Marang et al., 2014) and Antifoam 204 (Sigma Aldrich) (85 µL/L) were supplied to inhibit nitrification and foaming, respectively.

The reactor was controlled with the Iris Software (INFORS HT) monitoring online the dissolved oxygen concentration (pO₂). Periodically, a whole cycle was fully characterized by harvesting offline samples. The stability of the reactor was assessed based on the duration of the feast and famine phases (determined by the pO₂ profile) and PHA content at the end of the feast phase.

2.2.2 *Enrichment under nitrogen limitation during the feast phase (SBR_noN)*

SBR_noN was operated under nitrogen limitation during the feast phase and nitrogen availability during the famine phase (Fig. 1B). The reactor was inoculated from the culture from SBR_N and was initially operated with a SRT and HRT of 4 days, where one fourth of the volume of the reactor (425 mL) was replaced with new medium every 24h. Nevertheless, after cycle 24, the cycle length was adjusted back to 12 h as in SBR_N, given that 1,3-PDO consumption time decreased, resulting in a HRT and SRT of 2 days and the same OLR as SBR_N. Aeration, agitation, temperature, and pH were maintained as described for SBR_N, as well as a substrate concentration at 45 C_{mM} at the beginning of the cycle.

In the medium, the C:N:P ratio was 100:0:1.5. Nitrogen was supplied in the famine phase by means of a control algorithm based on the slope of the pO_2 saturation curve, given that a sharp increase was observed after substrate consumption. Addition of nitrogen in the form of NH_4Cl started at 30 mg/L NH_3-N . However, in order to provide the precise amount of nitrogen needed, the residual nitrogen at the end of the cycle was monitored and the concentration of NH_3-N was progressively corrected to 40 mg/L.

2.3 Batch PHA accumulation assays

PHA accumulation assays were performed in batch mode using the effluent from the respective SBR at the end of the cycle as inoculum.

When the performance on fermented effluent was evaluated (section 3.1.2), the same medium as for the SBR_N was prepared (45 C_{mM} at time 0), but without addition of nitrogen. As in the SBR, 850 mL of culture and 850 mL of medium were used. Alternatively, when individual substrates were evaluated (sections 3.1.1 and 3.2.1), the BA growth medium supplemented with butyrate or 1,3-PDO to the same concentrations as those in the fermentation effluent were used. In these experiments, the culture from the SBR was centrifuged and resuspended in medium to avoid any interference from the effluent matrix. In order to keep the same ratio of [culture]:[total volume] as in the SBR, 850 mL of culture were centrifuged and re-suspended in 1700 mL total volume for SBR_N (section 3.1.1), while 425 mL of culture were centrifuged and re-suspended in 567 mL total volume for SBR_noN (section 3.2.1).

Phosphorous, allylthiourea and antifoam were supplied to the concentrations described for the SBR. Temperature, pH, agitation and aeration were also controlled as in the SBR.

2.4 PHA extraction

PHA was extracted from 1 g of lyophilized cells with 50 mL of chloroform at 65°C for 4 h with frequent manual mixing. The suspensions were then filtered through 0.22 μm PTFE membranes and PHA was precipitated from the solution by dropwise addition of methanol under stirring (ratio methanol:chloroform 10:1) and finally recovered in 0.7 μm glass fiber filters.

2.5 Analytical methods

Offline samples of the reactors were analyzed in the following manner. OD_{600} , total soluble solids (TSS) and Nile Blue fluorescence were assessed in duplicates in fresh samples. Nile Blue was measured according to the protocol of Oshiki et al., (Oshiki et al., 2011) as an indirect indicative measurement of the PHA

concentration. TSS were measured according to standard protocols using Whatman filters GF/F (APHA et al., 2005).

A sample of approximately 50 mL was then centrifuged for 10 min at 4000 g and the supernatant was filtered through 0.45 μm filters. VFA (acetate, propionate, lactate, iso and *n*-butyrate and iso and *n*-valerate) and alcohols (1,3-PDO, ethanol, butanol and glycerol) in the supernatant were analyzed by HPLC in a Shimadzu HPLC system equipped with an RI detector and an Aminex HPX-87H column (BioRad). Soluble nitrogen in the form of ammonia (N-NH_3) and soluble COD were determined using Hach-Lange kits LCK 305/303 and LCK 914/514, respectively. Soluble inorganic reactive phosphorous was determined photometrically by the ascorbic acid method (APHA et al., 2005) in a plate reader.

Cell pellets after centrifugation were washed with 10 mL of PBS buffer and freeze-dried overnight. Lyophilized cell pellets were used for the determination of PHA and glycogen, both performed in duplicates. The glycogen content in the cell pellets was assessed using the protocol optimized for mixed cultures in the study of Lanham et al. (Lanham et al., 2012): 2 mL of 0.9 M HCl were added to 2 mg of freeze-dried cells and incubated at 100°C for 3 h in a block heater. The glucose content was then analyzed by HPLC as mentioned above.

The PHA content of the cells was assessed according to the protocol described in Johnson et al., (2009) with minor modifications: 10 mg of freeze dried cell pellets were dissolved in 1.5 mL of 1,2-dichloroethane (DCE) and 1.5 mL of HCl:1-propanol solution (1:4 v/v). 25 μL of a solution of 40 mg/mL of benzoic acid in 1-propanol were added as internal standard. The mixture was incubated for 2 h at 100°C in a block heater. After incubation, 3 mL of an 80 g/L NaHCO_3 solution were added to induce phase separation and neutralize the acidity. 1.7 mL of the organic phase were transferred into an Eppendorf tube with 0.2 g of sodium sulfate to absorb residual water and filtered through 0.2 μm PTFE filters. Samples were then analyzed in a GC-FID system (Agilent 6890) equipped with a SGE BP21 column. Quantification was done with commercial poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) and 3-hydroxypropionic acid (Sigma Aldrich) submitted to the same assay.

Nuclear magnetic resonance spectroscopy (NMR) on the chloroform extracted polymer was performed on a 300 MHz Cryomagnet from Spectrospin & Bruker (^1H -NMR at 300MHz, ^{13}C -NMR at 75MHz), at room temperature. Fourier Transform Infrared spectroscopy (FT-IR) was performed on a Thermo-Fischer is50 FT-IR with a universal attenuated total reflection (ATR) sampling accessory on a diamond crystal.

2.6 Calculations of yields and rates

The concentration of active biomass (cells excl. PHA) was obtained by subtracting the PHA and the glycogen from the TSS concentration. A formula of $C_5H_7NO_2$ was considered for the cells (Serafim et al., 2004). Substrate consumption rates ($-r_s$) (C_{mM} S/h) and product formation rates (r_p) (C_{mM} PHA/h) were calculated from the slope of the function of concentration vs. time. Likewise, specific substrate consumption rates ($-q_s$) (C_{mol} S/ C_{mol} X.h) and specific product formation rates (q_p) (C_{mol} PHA/ C_{mol} X.h) were calculated with the function of ratio of the substrate or product concentration to the respective biomass concentration at each time point. The value of the rates was the slope of the function of the ratio vs. time.

The yields of PHA ($Y_{P/S}$) (C_{mol} PHA/ C_{mol} substrate) and glycogen ($Y_{gly/S}$) (C_{mol} Glycogen/ C_{mol} substrate) were calculated by dividing the amount of polymer formed by the amount of substrate consumed. Biomass yield ($Y_{X/S}$) (C_{mol} X/ C_{mol} substrate), where calculated in the same way, where X refers to the active biomass (TSS excluding PHA and glycogen). Yields and rates reported in terms of C_{mol} were obtained from the compounds measured by HPLC, while yields reported in terms of COD_{total} were obtained with the measurements of the soluble COD in the supernatants.

2.7 Microbial community analysis

At certain time points, 10 mL of culture were withdrawn from the reactors and frozen at -20°C . Culture s were thawed at 4°C for 5 h, centrifuged at 4000 g for 10 min prior to freeze-drying the pellet. DNA extraction was performed using the ZR Soil Microbe DNA MiniPrepTM (ZymoResearch) and DNA concentrations were determined using the Qubit® dsDNA BR Assay Kit (ThermoFisher Scientific). 16S rRNA gene amplicon sequencing was basically performed according to the procedures described previously (Varrone et al., 2015), except that version v.2.5.1 of the MiSeq Reporter Software was used. Comparisons between samples were done at the genus level. Sequencing reads have been deposited to the Sequence Read Archive of NCBI under the BioProject ID PRJNA389518.

3 Results

3.1 Enrichment with nitrogen availability (SBR_N)

Using the effluent from the fermentation as substrate, an SBR was established (SBR_N) to enrich a population able to produce PHA from VFA and 1,3-PDO (Fig. 1A). Initially, a molar ratio C:N:P of 100:8:1 was applied. With this ratio, nitrogen and phosphorous were depleted before the end of the cycle, which could

have compromised the fate of the enrichment, given that the nutrients were not available to enable full growth from the PHA during the famine phase (Albuquerque et al., 2007). Consequently, the molar ratio of C:N:P of the enrichment was increased to 100:12:1.5 from cycle 19.

After the C:N:P ratio was increased, the feast phase for 1,3-PDO decreased considerably (Fig. 2A), and after a few cycles of fluctuations, it was stabilized at around 4 h. Likewise, the time needed to consume VFA settled at around 1.5 h. These would represent a feast/famine ratio of 0.1 and 0.4 for butyrate and 1,3-PDO, respectively, which are in the range of values allowing PHA accumulation reported in literature (Albuquerque et al., 2010).

A cycle of the SBR_N after stabilization of the feast phase duration (cycle 109) is presented in Fig. 3 (first column of graphs). PHA production was observed from the VFA (up to 20% of the TSS) (Fig. 3D), leading to HB (3-hydroxybutyrate) and HV (3-hydroxyvalerate) monomers, most probably coming from butyrate and propionate, respectively. Glycogen did not vary significantly during the cycle and accounted for up to 5% of the cell dry weight. No net production of PHA from 1,3-PDO was observed when only this substrate was present in the medium (after 2h) and only cell growth was observed (Fig. 3C). The depletion of VFA and 1,3-PDO was clearly distinguished by a sharp increase in the oxygen saturation of the broth (Fig. 3A).

Besides VFA and 1,3-PDO, the substrate also accounted for around 300 mg/L of undefined COD, meaning the difference between the total COD measured in the sample and the sum of the metabolites identified in the supernatant by HPLC. Part of this undefined COD was consumed simultaneously to the known substrates (Fig 3 B). No further consumption occurred during the famine phase, thus not affecting the feast-famine regime. The presence of undefined COD was most probably derived from the presence of residual fats and oils in crude glycerol. Methanol was not expected to contribute significantly to the undefined COD fraction, as its initial concentration was estimated to be around 25 mg/L based on methanol concentration in the crude glycerol (Varrone et al., 2015).

Nitrogen was consumed mostly during the feast phase (Fig. 3B), coinciding in time with cell growth. In regard to the Nile Blue fluorescence (Fig. 3D), used as a rapid indication of the PHA concentration, a similar tendency to the PHA measured by GC chromatography was observed.

3.1.1 PHA accumulation with synthetic substrates

PHA production from 1,3-PDO was not observed in the enrichment cycles. Nevertheless, it could be that this transformation was masked by a simultaneous consumption of the PHA produced from VFA. In order to rule that out, a series of batch experiments with synthetic substrates were performed.

PHA accumulation from butyrate and 1,3-PDO was tested in the concentrations present in the fermented effluent, using the culture of the enrichment (SBR_N). Both substrates were tested in the presence and absence of nitrogen. In the presence of nitrogen, only butyrate was transformed into PHA (entirely PHB), while 1,3-PDO mainly led to biomass growth (Fig. 4B and E). The yield obtained on butyrate was $0.51 \text{ C}_{\text{mol}} \text{ PHA} / \text{C}_{\text{mol}} \text{ butyrate}$ (Table 1).

In the absence of nitrogen, both substrates produced PHA to a final polymer percentage of around 10% of the cell dry weight (Fig. 4 I and L). Butyrate produced exclusively PHB, while 1,3-PDO led to trace amounts of HV and HP (3-hydroxypropionate) (less than 0.5%) besides HB. The PHA yield was higher for butyrate than for 1,3-PDO, with the respective values being 0.28 and $0.15 \text{ C}_{\text{mol}} \text{ PHA} / \text{C}_{\text{mol}} \text{ substrate}$ (Table 1). Noteworthy, a slight glycogen production was also observed when 1,3-PDO was used as substrate (Fig. 4I). The PHA yield on butyrate was lower in the absence of nitrogen than in the presence of nitrogen (0.51 vs. $0.28 \text{ C}_{\text{mol}} \text{ PHA} / \text{C}_{\text{mol}} \text{ butyrate}$, respectively) (Table 1). In all cases, the Nile Blue fluorescence presented a similar tendency to the PHA detected by GC.

For both substrates, the specific substrate uptake ($-q_s$) was faster when nitrogen was available. In the absence of nitrogen, the rate in 1,3-PDO was approximately the same compared to that with butyrate. (0.12 vs. $0.11 \text{ C}_{\text{mol}} \text{ S} / \text{C}_{\text{mol}} \text{ X.h}$) (Table 1), despite that in the experiment with 1,3-PDO the substrate was consumed in 8 h.

3.1.2 PHA accumulation with fermentation effluent under nitrogen limiting conditions

Results obtained with synthetic substrates implied that in the SBR_N, where nitrogen was available, no PHA from 1,3-PDO was being produced. Nevertheless, the culture had the capacity to produce PHA from both substrates in the absence of nitrogen (see above). Therefore, a batch PHA accumulation experiment was conducted in nitrogen limiting conditions with the culture enriched in the SBR_N and fermentation effluent as substrate. Results are presented in Fig. 3 (E-G) and Table 1 together with the data obtained during a cycle of the SBR_N for comparison.

Despite the limitation of nitrogen, still no PHA production was observed when 1,3-PDO was the only substrate in the fermentation (Fig. 3G). However, differently to what had been observed in all previous experiment, an important discrepancy between the measured PHA and the Nile Blue fluorescence was observed (Fig. 3G).

Lower substrate consumption rates were observed compared to the SBR cycle (Table 1). Nonetheless, as in SBR_N, both substrates presented similar specific substrate consumption rates.

3.1.3 Confirmation of the polymer structure (enrichment SBR_N)

To further confirm that no other PHA than the ones detected by the GC analysis was being produced, a chloroform extraction was performed with the cells at the end of the batch experiments with synthetic substrates (1,3-PDO and butyrate without nitrogen) and at the end of the PHA accumulation with fermentation effluent. The NMR and IR analyses of the extracted material confirmed in all cases the structure of the PHA, showing the presence of HB and HV in the NMR, as well as clear ester stretches in the IR. Trace amounts of HP units were also confirmed in the experiment with 1,3-PDO without nitrogen (see spectra in the Appendix A).

3.2 Enrichment with nitrogen limitation during the feast phase (SBR_noN)

Experiments performed so far proved the capacity of the culture to produce PHA from 1,3-PDO only in absence of nitrogen. Nevertheless, during the enrichment in SBR_N, nitrogen was available. This would mean that the selection was being performed in conditions not promoting PHA production from 1,3-PDO. Thus, a new strategy was formulated consisting of an SBR where nitrogen was limited in the feast phase. Nitrogen was provided during the famine phase to enable growth of PHA accumulators out of the stored PHA (SBR_noN Fig. 1B).

Given that in the absence of nitrogen 1,3-PDO was consumed within 10 h (Fig. 3E), the reactor was operated at higher biomass concentrations, with cycles of 24 h but a SRT and HRT of 96 h, where only $\frac{1}{4}$ of the volume of the reactor was replaced at the end of each cycle. After around 15 cycles though, the 1,3-PDO consumption time started decreasing, and it was finally settled around 4 h, very similar to that obtained in SBR_N (Fig. 5A). For this reason, the cycle length was lowered again to 12 h after cycle 24, which provided practically the same feast/famine ratios as for SBR_N: 0.1 and 0.4 for VFA and 1,3-PDO, respectively.

A cycle of the SBR_noN reactor after stabilization is presented in Fig. 3 (last column of graphs). The most important observation was that, in this case, PHA concentration increased when 1,3-PDO was the only substrate left after VFA consumption, with HB being the main product. HV was only produced during the VFA consumption phase, and trace amounts of HP were observed from both substrates (less than 1% of the cell dry weight). Also, a slight increase in the glycogen content associated with 1,3-PDO consumption was observed in agreement with the experiments with synthetic substrates. PHA reached approximately 20% of

the cell dry weight during the VFA consumption phase and finally 23% after 1,3-PDO depletion (Fig. 3K). It is worth mentioning that these values are lower than those obtained in the previous enrichment just from VFA (Fig. 3G). Nevertheless, as this reactor had a higher cell concentration, the PHA concentration in the reactor broth was higher than that obtained in the previous enrichment (Fig. 3J).

As in a typical feast-famine cycle, the nitrogen supplied at the beginning of the famine phase was consumed to generate biomass from the PHA stored during the feast phase; this was also reflected in the pO_2 saturation curve (Fig. 3H). As in SBR_N, part of the unknown COD was consumed along with the depletion of VFA and 1,3-PDO, but approximately half of the undefined COD remained unconsumed after the feast phase (Fig 3 I).

Although 1,3-PDO was consumed simultaneously with the VFA and at similar rates (Table 2), the PHA yields and rates largely differed between the VFA and the 1,3-PDO consumption phases. When both substrates were present the PHA yield was $0.74 C_{mol} \text{ PHA}/C_{mol} \text{ S}$. This value decreased to 0.24 when 1,3-PDO was the only substrate.

In the supernatant of the samples from this experiment, increasing areas of a new peak were detected with the 1,3-PDO disappearance (Fig. 3I). The retention time of this peak coincided with the one of 3-hydroxypropionate (3-HP).

3.2.1 PHA accumulation in synthetic 1,3-PDO

In order to determine the net PHA yield from 1,3-PDO and rule out if PHA from VFA was masking the PHA production from 1,3-PDO, a new experiment using synthetic 1,3-PDO under nitrogen limiting conditions was established, using the culture enriched in SBR_noN. The PHA yield obtained in this experiment was $0.15 C_{mol} \text{ PHA}/C_{mol} \text{ S}$, thus lower than the one obtained when 1,3-PDO was the only substrate available in the real effluent, which was 0.24 (Table 2). As in the enrichment cycle, increasing concentrations of 3-hydroxypropionate were detected in the supernatant coinciding with the consumption of 1,3-PDO.

3.2.2 Confirmation of the polymer structure (enrichment SBR_noN)

Like in the previous enrichment, PHA was extracted from the enriched biomass collected just after 1,3-PDO consumption. NMR and IR of the isolated polymer confirmed the expected structure with repeated units of HB, HV and traces of HP, bonded by ester linkages (see spectrum in Appendix A). Signals corresponding to a possible 1,3-propanediol hydroxytermination of the polymer as described before for glycerol (Tanadchangsang and Yu, 2012) were not observed.

3.3 Microbial community analysis

Samples from both enrichment reactors were analyzed by 16S rRNA gene amplicon sequencing, which showed a very diverse population in both cases (Table 3) (see analysis at class and genus level in Appendix B). The community in SBR_N was dominated by bacteria from the genera *Xanthobacter*, *Rhodococcus*, *Paracoccus* and *Acinetobacter*, which together accounted for around 50% of the OTUs. SBR_noN presented a similar percentage of the genera *Paracoccus* and *Xanthobacter*, but *Rhodococcus* and *Acinetobacter* practically disappeared from the culture. Instead, two more genera appeared in high percentages: *Gordonia* (14.9%) and *Segetibacter* (9.2%).

Also a sample at the end of the PHA accumulation with synthetic 1,3-PDO in the presence of nitrogen (correspondent to the experiments presented in section 3.1.2) was analyzed, to obtain indications of the bacteria responsible for growth on 1,3-PDO in the SBR_N. *Rhodococcus* was the genus presenting a higher increase (from 14.1% to 24.4%) compared to the mother culture (SBR_N cycle 109). The genera *Tepidimonas* and *Hylemonella* also presented a high relative increase (108 % and 77%, respectively), although their abundance remained below 10%.

4 Discussion

4.1 The requirement of nitrogen limitation for the PHA production from 1,3-PDO

PHA is produced in the cells under conditions of unbalanced growth. This can mean a limitation in external factors such as nutrients (nitrogen or phosphorous) or electron acceptors (oxygen), as it is the case for the aerobic/anaerobic enrichments. Nevertheless, the limitation can also be internal, such as insufficient RNA or enzymes required for growth after a starvation period, with this being the most accepted mechanism for the PHA storage under aerobic dynamic feeding (Dias et al., 2006; Ren et al., 2009). The latter has proven to be effective in selecting PHA accumulators in mixed microbial consortia fed with many different substrates (Serafim et al., 2008). However, there are some strains able to produce PHA only under nutrient limiting conditions, such as *Cupriavidus necator*, the most well-known PHA producer (Lee, 1996). There is no evidence that the feast-famine strategy, which provides a competitive advantage for PHA accumulators, rather than imposing a limitation, is enough for the enrichment of PHA accumulators from all types of substrates.

A possible example of this can be observed in the first of the strategies evaluated in the present study (SBR_N), a classical feast and famine enrichment, where nitrogen was available during the whole cycle. In

this enrichment, 1,3-PDO contained in the fermentation effluent was not converted to PHA but mostly contributed to cell biomass increase (section 3.1). Moreover experiments performed in the presence of only 1,3-PDO or butyrate showed that only butyrate, but not 1,3-PDO, was converted to PHA when nitrogen was present in the medium (Section 3.1.2). On the other hand, when nitrogen was absent, both butyrate and 1,3-PDO were converted to PHA (from synthetic substrates). Thus, the results implied that, under the conditions of the enrichment, only the internal factor limitation was not enough to obtain PHA production from 1,3-PDO, and nitrogen limitation was also required.

Nonetheless, when nitrogen limitation was applied to this culture using effluent as a substrate, no net production of PHA from 1,3-PDO was observed when the latter was the only remaining substrate (just after the VFAs were up-taken) in the fermentation (section 3.1.3). As a matter of fact, the PHA degradation yield was the same as in the presence of nitrogen (-0.22 vs -0.21 C_{mol} PHA/ C_{mol} S) (Table 1). Consequently, it could not be concluded that PHA production from 1,3-PDO was masked by the degradation of the PHA produced from VFA. However, the PHA yield during the VFA consumption phase was higher than obtained in the presence of nitrogen (0.68 vs 0.56 C_{mol} PHA/ C_{mol} S) (Table 1), leading to almost 30% PHA in the cells at the moment of VFA depletion. This would not be justified with a higher PHA production from butyrate (which was observed to have a lower yield without nitrogen; see Table 1). Thus, it could be possible that certain PHA production from 1,3-PDO did occur. Yet, also other compounds present in the fermentation effluent could have been used as a substrate.

4.2 PHA production from 1,3-PDO in the enrichment without nitrogen during the feast phase (SBR_noN)

The presence of nitrogen during the SBR cycles is generally accepted to favour enrichment of PHA producers given that in this condition, bacteria can grow during the famine phase from the accumulated PHA, which otherwise is just used for maintenance (Albuquerque et al., 2007). On the other hand, PHA yields in the accumulation phase are higher in absence of nitrogen (Johnson et al., 2010b; Marang et al., 2014; Serafim et al., 2004). This fact, together with the above described production of PHA only from 1,3-PDO exclusively under nitrogen limiting conditions, justified a strategy where nitrogen was limited during the feast phase and supplied in the famine phase for the growth of PHA accumulating bacteria (SBR_noN Fig.1B).

This enrichment strategy enabled a net production of PHA once VFA had depleted and only 1,3-PDO was present in the medium. The conversion had a low yield (0.24 C_{mol} PHA/ C_{mol} S or 0.21 g COD_{PHA}/g COD_{total}), which could not be attributed to the simultaneous degradation of the PHA produced from VFA since the

experiment with only 1,3-PDO as a substrate gave an ever lower yield of $0.15 \text{ C}_{\text{mol}} \text{ PHA/C}_{\text{mol}} \text{ S}$ or $0.13 \text{ g COD}_{\text{PHA}}/\text{g COD}_{1,3\text{-PDO}}$ (Table 2). The higher yield in fermented effluent could be partially explained by the contribution of residual COD to the PHA production. As a matter of fact, certain undefined COD was also consumed during this phase (Fig.3I). Remarkably though, this COD alone (70 mg/L) would not explain an increment of 213 mg COD/L in PHA. Thus, 1,3-PDO was certainly contributing to the PHA production.

4.2.1 3-hydroxypropionate (3-HP) as possible intermediate

The culture enriched without nitrogen during the feast phase (SBR_noN) showed 3-HP increase along with the degradation of 1,3-PDO, both when using fermentation effluent and with synthetic 1,3-PDO. 3-HP-CoA was suggested as an intermediate of 1,3-PDO conversion to PHA in a *Chromobacterium* strain producing a mixture of medium and long chain PHA (Kimura et al., 2002) (Fig. 6). Interestingly, the accumulation of intermediates is believed to be a key regulation mechanism for PHA production in certain species that require nitrogen limitation (Kojima et al., 2004).

The fact of 3-HP being detected in the supernatant suggested that 3-HP may be excreted, and allows for hypothesizing a cross-feeding phenomenon, where 3-HP could be transformed to PHA by other species than the ones degrading the 1,3-PDO. Moreover, co-metabolism effects could also be occurring in the culture, which could contribute to explain the lower PHA yield obtained in pure synthetic 1,3-PDO compared to the one obtained in the fermentation effluent (section 3.2.2). More explicitly, it could be proposed that in real effluent, 3-HP excreted into the medium was converted into PHA by other bacteria, producing as well PHA from VFA. In the absence of VFA, the PHA production enzymes might not be induced, thus lowering the yield of PHA.

Differences in the behavior of cultures when submitted to mixed substrates or single substrates have been previously described in studies combining the labelling of substrates and microbial strains with MAR and FISH (Albuquerque et al., 2013), where co-metabolism effect were also suggested.

Is it worth emphasizing the fact that in both enrichments, HB was the main monomer produced from 1,3-PDO, while only traces of HP and HV were detected. On the contrary, whenever 1,3-PDO conversion had been reported, HP contributed significantly to the polymer fraction (Kimura et al., 2002), following the pattern that odd carbon substrates lead to odd-carbon monomers (Dias et al., 2006) (Fig. 6). This observation might support the suggestion that in the present study 1,3-PDO was not directly converted to PHA in the same microorganism.

4.2.2 Further advantages of the enrichment without nitrogen

Apart from enabling the conversion of 1,3-PDO to PHA, this type of enrichment could also imply that a separate PHA accumulation phase would no longer be needed, given that the enrichment itself would be performed at the conditions maximizing the PHA production. If part of the biomass was removed from the reactor at the end of the feast phase, cells with maximum PHA content could be obtained without the need of an extra reactor (Fig. 1B). Needless to say, the viability of this configuration would be conditioned to an increment of the PHA content of the cells at this stage by incrementing the substrate load as discussed in section 4.4.

4.3 Insights into the microbial community

4.3.1 The presence of lipid accumulating bacteria

The most predominant genera identified in the reactors were *Xanthobacter*, *Rhodococcus*, *Paracoccus*, *Acinetobacter*, *Gordonia*, and *Segetibacter*. All of them except *Segetibacter* (a relatively undescribed genus) have been previously detected, in higher or lower frequency, in SBR working under feast-famine regimes for PHA production (Morgan-Sagastume, 2016). Moreover, representatives of all the genera, except *Segetibacter*, have been directly linked to PHA accumulation (Alvarez, 2003; Kojima et al., 2004; Schembri et al., 1995; Wiegel, 2006).

It is important to notice though, that *Rhodococcus*, *Acinetobacter* and *Gordonia*, are especially well known for their capacity to accumulate internal lipid inclusions, such as wax esters and triacylglycerols. This ability has been reported from various carbon sources such as organic acids and alcohols, particularly under nitrogen limiting conditions (Alvarez and Steinbüchel, 2002). In contrast to PHA synthesis, which is widespread among prokaryotes, the capacity to synthesize lipid inclusions is limited to a very narrow spectrum of bacterial genera. Consequently, their presence should not be disregarded. Moreover, the metabolism of lipid storage compounds and PHA is largely interconnected, given that both are produced as storage compounds under unbalanced growth conditions and use acyl-CoA molecules as intermediates (Alvarez and Steinbüchel, 2002; Thomson et al., 2010) (Fig. 6). As a matter of fact, Kimura and colleagues suggested that malonyl-CoA, the first metabolite in the fatty acid biosynthesis, could be directly formed from propionyl-CoA in the oxidation of 1,3-PDO, suggesting a direct link to the fatty acid biosynthesis. Thus, the accumulation of lipids instead of PHA could be a possible explanation of the low PHA yields obtained from 1,3-PDO.

Further evidence of the accumulation of lipids could be found in the results obtained from the Nile Blue analysis. This compound has typically been used for screening of PHA-producing bacteria and even

suggested as a rapid method of quantification of PHA (Oshiki et al., 2011). Nevertheless, it can also bind to lipid inclusions, given that the interaction is due to hydrophobicity (Spiekermann et al., 1999). In the experiments with pure substrates (Fig. 4), PHA and Nile blue followed similar trends, as well as in the fermentation effluent in the presence of nitrogen (Fig. 3D). Nonetheless, in the experiment with fermented effluent in the absence of nitrogen (Fig. 3G), a significant difference was observed in the curves of PHA and Nile Blue, which could be attributed to Nile Blue binding to lipid inclusions.

Given the known interactions of Nile Blue to lipid inclusions, its use in PHA quantification should be carefully considered.

4.3.2 Genera related to 1,3-PDO consumption

Most of the main genera described in the reactors (*Xanthobacter*, *Rhodococcus*, *Paracoccus*, *Acinetobacter* and *Gordonia*) are known to be able to use various alcohols as carbon sources (De Carvalho and Da Fonseca, 2005; Eberly et al., 2013; Kojima et al., 2004; Singer and Finnerty, 1985; Wiegand, 2006). Thus, the characteristics of the communities itself did not give further insights into the substrate consumption preferences.

The main changes occurring with the different enrichment strategies corresponded to the reduction of OTUs corresponding to the genera *Rhodococcus* and *Acinetobacter*, together with the appearance of OTUs classified as *Gordonia* and *Segetibacter* when nitrogen was limited (Table 3). As the most important change in the behavior of the culture occurred in terms of 1,3-PDO conversion, it could be suggested that those genera were the ones involved in the 1,3-PDO consumption. A further indication of this could be the fact that *Rhodococcus* was the genus increasing more in abundance with respect to the mother culture when only 1,3-PDO was supplied in the presence of nitrogen. As no PHA was detected in that case, the assumption could be that 1,3-PDO was being used mostly for growth in the members of this genus, whose presence was dramatically reduced when nitrogen was limited in SBR_{noN}.

Furthermore, also *Tepidimonas* and *Hylemonella*, increased considerably their relative abundance when only 1,3-PDO was present in the medium. Thus, these genera could also have been contributed to the utilization of 1,3-PDO for growth. Like *Rhodococcus*, their presence was reduced in the reactor operating under nitrogen limitation (SBR_{noN}).

4.4 Overall discussion on utilization of 1,3-PDO to produce PHA

A moderate PHA yield was obtained from 1,3-PDO in the fermented effluent ($0.24 \text{ Cmol PHA/Cmol S}$). As discussed above, this yield could be partially explained by the carboxylation step occurring in its conversion to acetyl-CoA (Fig 6. A), but was also limited by a flow of carbon to glycogen, cell biomass and possibly lipid inclusions. Thus, even though this study represents an important step in demonstrating the possibility of the transformation of 1,3-PDO into PHA, further investigation is needed to enhance the feasibility of the process. Along this line, it would be important to elucidate the competition between PHA and other storage polymers, and study the operational parameters that maximize PHA production. Such parameters have been observed to have a very important influence in the carbon distribution between glycogen and PHA in other studies (Freches and Lemos, 2017). Likewise, higher substrate feedings should be tested in order to achieve higher productivities and PHA content. Only low OLRs were tested in this study in order to limit inhibition phenomena in the process of studying a novel substrate. Possible inhibition phenomena resulting from the presence of long-chain fatty acids and/or methanol should be addressed when testing higher substrate concentrations. Moreover, it would be interesting to study the fate of 1,3-PDO within the culture by means of combined MAR and FISH to elucidate possible cross-feeding phenomena and the implications of 3-hydroxypropionate excretion.

In terms of overall yields, the PHA yield obtained from the fermentation effluent in the present study was $0.42 \text{ Cmol PHA/Cmol S}$, which, taking into account the stoichiometry of the fermentation step, corresponded to $0.36 \text{ Cmol PHA/Cmol glycerol}$ ($0.31 \text{ g COD}_{\text{PHA}}/\text{g COD}_{\text{total}}$). This yield is slightly lower than the maximum obtained from crude glycerol in mixed cultures ($0.36\text{-}0.51 \text{ Cmol PHB/Cmol S}$) (Freches and Lemos, 2017), but similar to reported yields in terms of COD ($0.32 \text{ g COD/g COD}_{\text{total}}$ in Moita et al., 2014). Thus, this study proved the possibility of an alternative strategy with similar perspectives in terms of yields. Moreover, the use of glycerol as a substrate leads to only PHB, while the polymer obtained in this study contained both HB and HV monomers. Such copolymers have been reported to have more desirable properties as a biopolymer than PHB, such as higher flexibility and tensile strength (Laycock et al., 2014). Furthermore, the PHA composition and yield can be further modulated by studying the distribution of metabolites depending on the fermentation operational conditions.

5 Conclusions

The present study investigated the possibility of producing PHA from a crude glycerol fermentation effluent with volatile fatty acids and 1,3-propanediol as main substrates, focusing on the potential of 1,3 PDO to be up-taken for PHA production. The following conclusions could be extracted:

- The conventional aerobic dynamic feeding strategy with nitrogen availability during the whole cycle failed to produce PHA from 1,3-PDO in the fermentation effluent, under the tested conditions.
- Experiments with individual synthetic substrates (butyrate and 1,3-PDO) showed the capability of the culture to produce PHA from 1,3-PDO only under nitrogen limiting conditions, proving the relevance of nitrogen limitation for this conversion.
- PHB was the main polymer produced from 1,3-PDO under nitrogen limiting conditions.
- 1,3-PDO conversion to PHA in the fermentation effluent was attained by means of an enrichment strategy that limited nitrogen during the feast phase and provided nitrogen during the famine phase, with an overall yield of $0.42 \text{ C}_{\text{mol}} \text{ PHA/C}_{\text{mol}} \text{ substrate}$ (including both VFA and 1,3-PDO). The yield when 1,3-PDO was the only substrate was $0.24 \text{ C}_{\text{mol}} \text{ PHA/C}_{\text{mol}} \text{ substrate}$. Noteworthy, this is, to our best knowledge, the first time that conversion yields from 1,3-PDO are reported.
- The two enrichment strategies led to important differences in the microbial community composition.
- Besides PHA, glycogen accumulation was also detected. Moreover, indications of lipid accumulation were also found. Thus, multiple storage responses seemed to be competing in the culture.

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Supplementary data

Appendix A contains the ^1H -NMR and IR spectrum of PHA extracted from the different enrichments.

Appendix B contains supplementary results of the microbial community analysis.

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Table 1: Yields and rates obtained with the culture SBR_N (enrichment with nitrogen), both in PHA accumulation experiments with synthetic substrates and fermentation effluent (with and without nitrogen). $Y_{P/S}$: yield PHA/substrate $Y_{X/S}$: Yield biomass/substrate $-r_s$: substrate consumption rate r_p : product formation rate q_p : Specific product formation rate $-q_s$: Specific substrate uptake rate

Substrate	Phase*	Nitrogen	Yields			Rates		Specific rates			
			$Y_{P/S}$	$Y_{P/S}$	$Y_{X/S}$	$-r_s$	r_p	$-q_s$			q_p
			$C_{mol} \text{ PHA} / C_{mol} S$	$g \text{ COD PHA} / g \text{ COD}_{total}$	$C_{mol} X / C_{mol} S$	$C_{mM} S/h$	$C_{mM} \text{ PHA}/h$	$C_{mol} S / C_{mol} X \cdot h$			$C_{mol} \text{ PHA} / C_{mol} X \cdot h$
								1,3-PDO	Butyrate	Overall	
Fermentation effluent	VFA	Yes**	0.56	0.46	0.36	17.69	9.98				
	1,3-PDO		-0.21	-0.17	0.88	9.17	-2.03	0.27	0.27	0.78	0.28
	VFA***	No	0.68	0.55	0.30	11.54	7.84				
	1,3-PDO		-0.22	-0.18	0.76	2.32	-0.50	0.08	0.11	0.48	0.22
1,3-PDO		Yes	0.01		0.43	5.70	0.00	0.24			0.00
		No	0.15		0.03	2.65	0.43	0.12			0.02
Butyrate		Yes	0.51		0.18	8.85	4.55		0.32		0.15
		No	0.28		-0.32	2.41	0.66		0.11		0.03

*The values were reported for the feast phase of the batch. For the fermentation effluent, VFA and 1,3-PDO phase refer to the interval where VFA were consumed and the interval where only 1,3-PDO was left, respectively.

**The values for this condition were obtained from cycle 109 of SBR_N.

*** The values corresponding to this phase were calculated accounting for an initial concentration of 1,3-PDO of 27.6 C_{mM} . Determination of 1,3-PDO at time 0 in Fig.3 E was considered to be a sampling error.

Table 2: Yields and rates obtained with the culture SBR_noN (enrichment without nitrogen), both using fermentation effluent (enrichment cycle 44) and in synthetic 1,3-PDO medium as substrates. Both experiments were performed under nitrogen limitation. $Y_{P/S}$: yield product/substrate $Y_{gly/S}$: Yield glycogen/substrate $Y_{X/S}$: Yield biomass/substrate $-r_s$: substrate consumption rate r_p : product formation rate q_p : Specific product formation rate $-q_s$: Specific substrate uptake rate

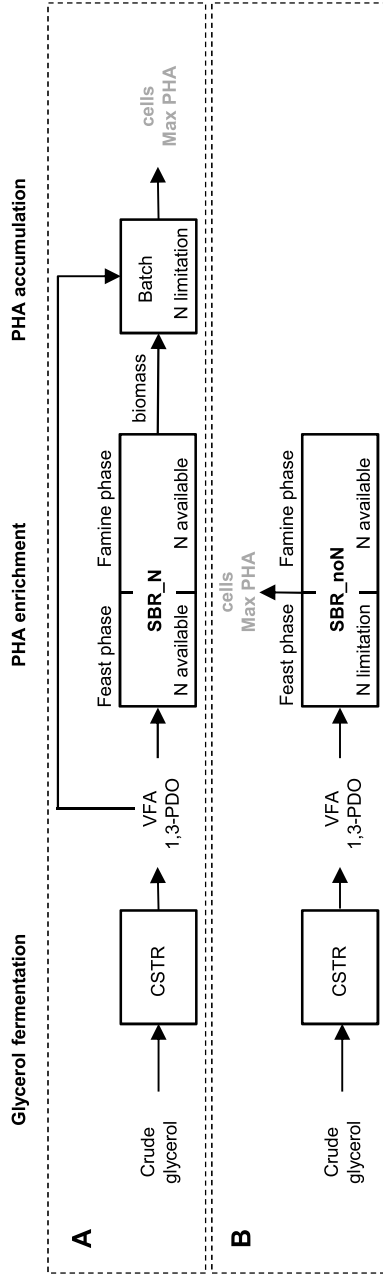
Substrate	Phase*	Yields				Rates		Specific rates			
		$Y_{P/S}$	$Y_{P/S}$	$Y_{gly/S}$	$Y_{X/S}$	$-r_s$	r_p	$-q_s$			q_p
		$\frac{C_{mol} \text{ PHA}}{C_{mol} \text{ S}}$	$\frac{g \text{ COD PHA}}{g \text{ COD}_{total}}$	$\frac{C_{mol} \text{ Glycogen}}{C_{mol} \text{ S}}$	$\frac{C_{mol} \text{ X}}{C_{mol} \text{ S}}$	$C_{mM} \text{ S/h}$	$C_{mM} \text{ PHA/h}$	$\frac{C_{mol} \text{ S}}{C_{mol} \text{ X.h}}$			$\frac{C_{mol} \text{ PHA}}{C_{mol} \text{ X.h}}$
								1,3-PDO	Butyrate	Overall	
Fermentation effluent	Overall	0.42	0.35	0.08	0.14	9.82	4.12**			0.17	0.06**
	VFA	0.74	0.56	0.05	0.09	14.37	10.57		0.11	0.26	0.18
	1,3-PDO	0.24	0.21	0.10	0.17	8.30	1.99	0.14		0.14	0.02
1,3-PDO		0.15		0.09	0.03	9.14	1.30	0.15			0.02

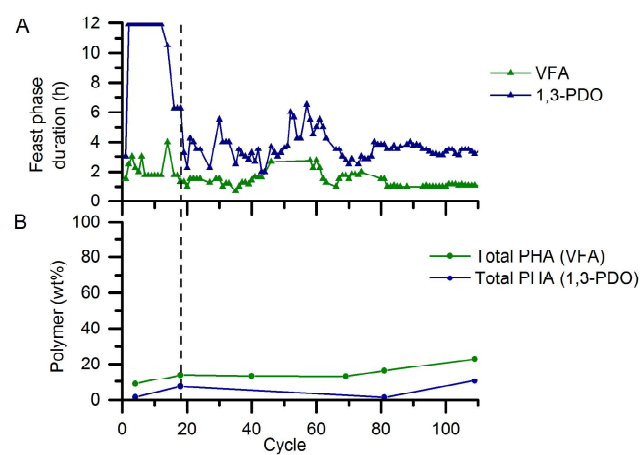
*The values were reported for the feast phase of the batch. For the fermentation effluent, VFA and 1,3-PDO phase refers to the interval where VFA were consumed and the interval where only 1,3-PDO was left, respectively. The term overall refers to the entire feast phase.

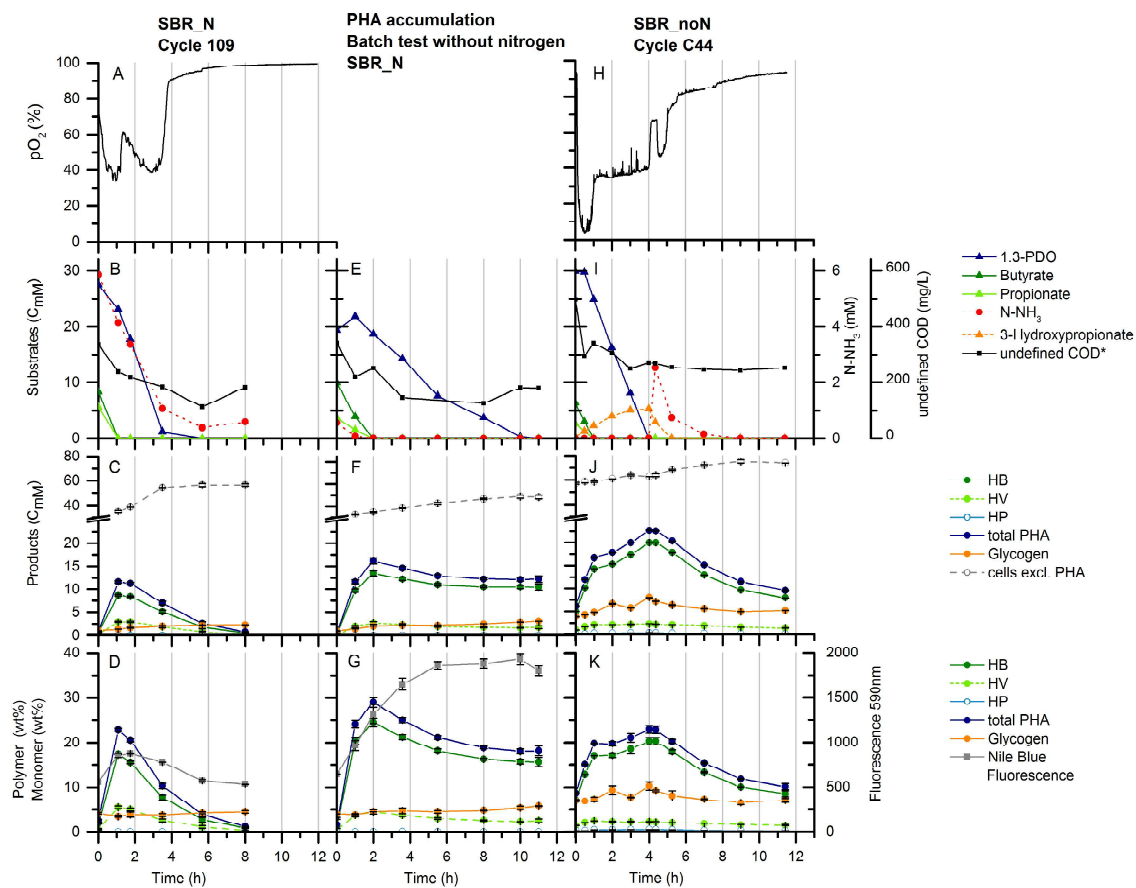
** Product rates for the overall feast phase could not be calculated from the slope of the experimental data because of the distinct behavior of both phases, thus these parameters were calculated dividing the increment in $C_{mM} \text{ PHA}$ or $C_{mM} \text{ PHA}/C_{mM} \text{ X}$ by the time interval.

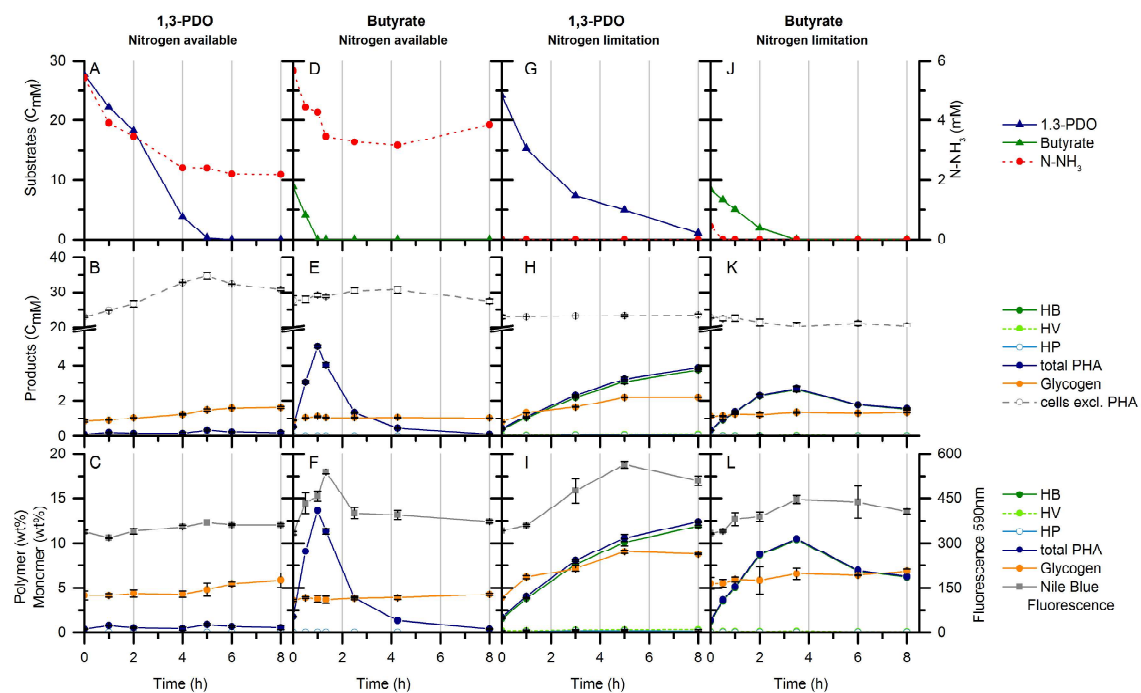
Table 3: Relative abundance of the predominant bacterial genera identified by 16S rRNA amplicon sequencing (> 1% in at least one of the samples). Grey scale intensity corresponds to relative abundance.

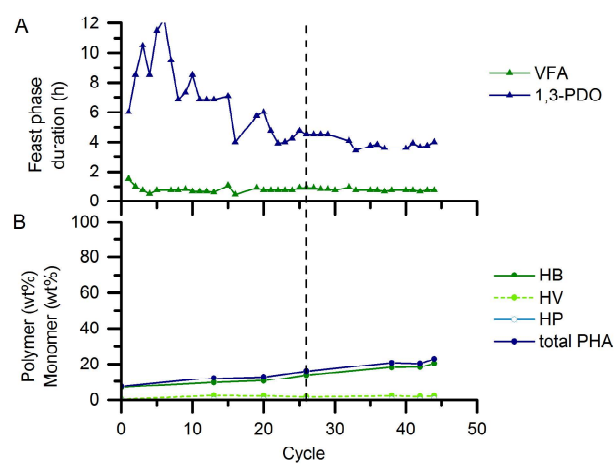
Enrichment reactor Sample	SBR_N		SBR_noN
	Cycle 109 (time 0 of the cycle)	1,3-PDO Nitrogen available (End of batch)	Cycle 44 (time 0 of the cycle)
Genera	OTU %		
<i>Xanthobacter</i>	15.3	6.0	13.0
<i>Rhodococcus</i>	14.1	24.4	2.1
<i>Paracoccus</i>	11.0	9.1	14.9
<i>Acinetobacter</i>	9.3	12.8	0.0
Unclassified	8.1	10.3	6.6
<i>Gordonia</i>	7.0	4.6	14.6
<i>Amaricoccus</i>	5.0	0.2	3.9
<i>Tepidimonas</i>	4.1	8.6	0.0
<i>Hylemonella</i>	2.8	4.9	1.6
<i>Leucobacter</i>	2.2	0.5	0.4
<i>Flavobacterium</i>	2.1	0.3	0.2
<i>Rhodobacter</i>	1.6	0.9	3.0
<i>Agrobacterium</i>	1.6	1.6	0.5
<i>Singulisphaera</i>	1.6	0.0	3.1
<i>Kaistella</i>	1.2	0.5	2.1
<i>Comamonas</i>	0.8	1.3	0.3
<i>Aquimonas</i>	0.7	0.0	1.0
<i>Shinella</i>	0.5	2.3	1.2
<i>Aminobacter</i>	0.4	1.7	0.5
<i>Pedobacter</i>	0.3	0.6	1.5
<i>Segetibacter</i>	0.0	0.0	9.2
<i>Sphingopyxis</i>	0.0	0.0	2.3
<i>Methylobacillus</i>	0.1	0.1	1.7
Others	10.1	9.1	16.3











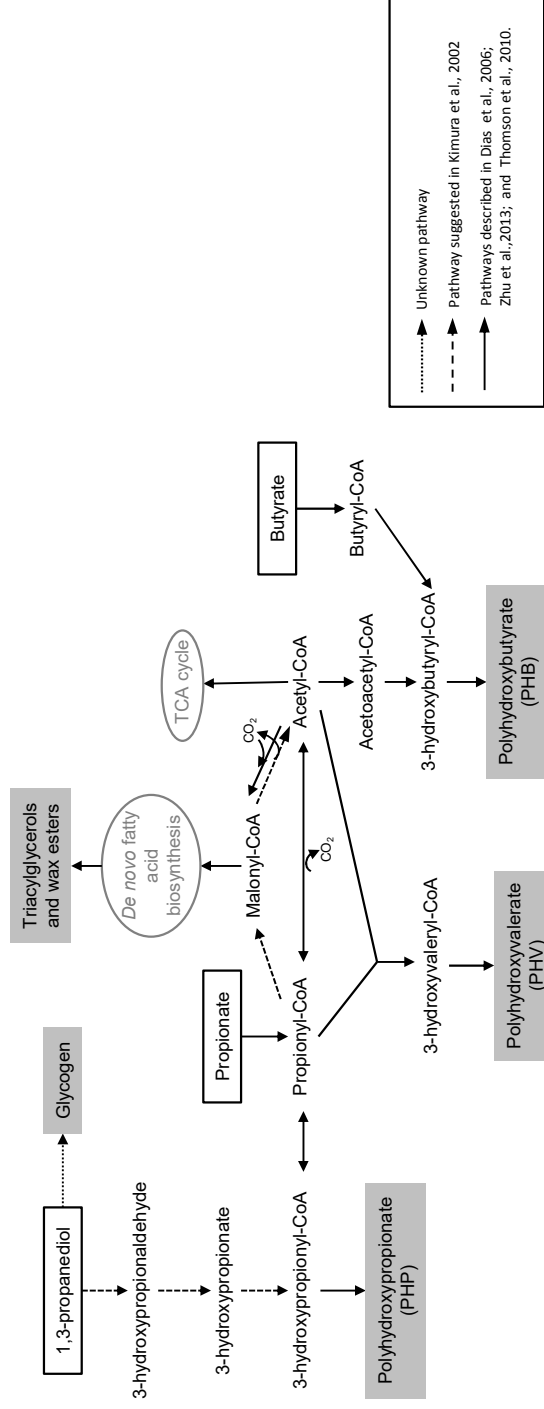


Fig. 1: Summary of strategies for production of PHA from fermented crude glycerol. CSTR: Continuous flow stirred-tank reactor; SBR: Sequential Batch Reactor.

Fig. 1: Evolution of the enrichment under nitrogen availability (SBR_N). A: Duration of feast phase (determined by pO_2 saturation). B: Polymer content in the cells (g polymer/100 g TSS) at the moment of VFA depletion and 1,3-PDO depletion. The dashed line indicates that from cycle 19, the molar ratio C:N:P in the feed was changed from 100:8:1 to 100:12:1.5.

Fig.3: Characterization of cycle 109 of the enrichment under nitrogen availability (SBR_N) (A-D), Batch PHA accumulation without nitrogen using the same culture (E-G), and cycle 44 of the enrichment with nitrogen limitation in the feast phase (SBR_noN) (H-K). A, H: profile of oxygen saturation. B, E, I: Concentration of substrates (C_{mm}). C, F, J: Concentration of products (C_{pm}). D, G, K: Polymer and monomer content in the cells (g/100 g TSS) and Nile Blue Fluorescence. * Undefined COD refers to the difference between the COD measured in the supernatant and the sum of theoretical COD of metabolites measured by HPLC.

Fig.4: PHA accumulation experiments with synthetic substrates. Each experiment corresponds to a column of graphs. A,D,G, J: Concentration of substrates (C_{mm}). B,E,H,K: Concentration of products (C_{pm}). C,F, I, L: Polymer and monomer content in the cells (g polymer/100 g TSS) and Nile Blue Fluorescence.

Fig.5: Evolution of the enrichment under nitrogen limitation during the feast phase (SBR_noN). A: Duration of feast phase (determined by pO_2 saturation). B: Polymer and monomer content in the cells (g/100 g TSS) at the moment of 1,3-PDO depletion. The dashed line indicates that from cycle 26, the cycle length was changed from 24h to 12h.

Fig.6: Schematic representation of the possible pathways involved in the conversion of 1,3-propanediol, butyrate and propionate into PHA, glycogen and other lipid inclusions.

Highlights

- Nitrogen had a critical role in the conversion of 1,3-propanediol (1,3-PDO) to PHA.
- An enrichment with nitrogen limitation during the feast phase was proposed.
- The PHA yield from 1,3-PDO was $0.24 \text{ C}_{\text{mol}} \text{ PHA} / \text{C}_{\text{mol}} \text{ substrate}$.
- The PHA yield from 1,3-PDO was limited by the synthesis of other storage compounds.